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PII: S0963-9969(19)30299-6
DOI: https://doi.org/10.1016/j.foodres.2019.04.067
Reference: FRIN 8448

To appear in: *Food Research International*

Received date: 12 November 2018
Revised date: 27 April 2019
Accepted date: 29 April 2019

Please cite this article as: L. Merino, F.M. Trejo, G. De Antoni, et al., Lactobacillus strains inhibit biofilm formation of Salmonella sp. isolates from poultry, Food Research International, https://doi.org/10.1016/j.foodres.2019.04.067

Lactobacillus strains inhibit biofilm formation of *Salmonella* sp. isolates from poultry

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Abstract

Lactic acid bacteria (LAB) exert a strong antagonistic activity against many microorganisms including food spoilage organisms and may be used as an alternative to control biofilm formation of pathogens in food industries. The objective of this work was to investigate the ability of fifteen *Salmonella* strains isolated from poultry environment to form biofilms on different surfaces. In addition, the effect of *Lactobacillus kefiri* strains 8321 and 83113 and *Lactobacillus plantarum* 83114 and their surface proteins on biofilm development of *Salmonella* Enteritidis 115 was studied. The relationship between surface properties of bacteria (hydrophobicity, autoaggregation and coaggregation with lactobacilli) and biofilm formation was also investigated. Most of *Salmonella* strains were hydrophilic and five strains were moderately hydrophobic. In general, *Salmonella* strains showed high aggregation abilities (27–54%). *S.* Enteritidis 106 and *S.* Typhimurium 102 and 108 showed the highest percentages of autoaggregation. All *Salmonella* strains tested showed aggregation abilities with the three lactobacilli studied, but the percentage of coaggregation proved to be strain-specific. When comparing stainless steel, glass and polystyrene surfaces, higher levels of biofilm formation occurred on polystyrene plate than on glass surfaces or stainless steel. *S.* Enteritidis 115 exhibited the greatest attachment to polyestyrene surface. The preincubation or coincubation with the three lactobacilli strains significantly reduced (about 1 log CFU/ml of reduction) the

ability of *S.* Enteritidis 115 to form biofilm compared to the control without lactobacilli. These results were confirmed by confocal microscopy. In the same way, when surface proteins extracted from lactobacilli strains were preincubated or coincubated with *S.* Enteritidis 115, biofilm formation of this strain was significantly decreased compared to the control. The results obtained showed that these *Lactobacillus* strains and their surface proteins can be used as alternatives for control of biofilm formation by *Salmonella* in the poultry industry.

Keywords: biofilm, biocontrol, aggregation, surface proteins

1. Introduction

Salmonella is a genus of rod-shaped (bacillus) gram-negative bacteria that represents an important global public health problem, causing substantial morbidity, and thus also has a significant economic impact (Sharma & Carlson, 2000). It consists of more than 2500 serologically distinguishable variants (or serotypes) that are frequently named for the place of initial isolation. In poultry, the numerous motile and non-host-adapted *Salmonella* serotypes, referred as paratyphoid *Salmonella*, are found nearly ubiquitously in wild and domestic animals. This diverse group of serotypes is principally of concern as a cause of food-borne disease in humans (Gast, 2008). *Salmonella* is capable of adhering and forming biofilms on both biotic and abiotic surfaces in the food processing environment (Joseph et al., 2001; Chia et al., 2009; Marin et al., 2009). According to various definitions that exist, a biofilm is an assemblage of microbial cells, irreversibly attached (not removed by gentle rinsing) to a surface growing in community and enclosed in a self-synthesized matrix primarily consisted of polysaccharide material (Steenackers et al., 2012). *Salmonella* spp. can form biofilms on food contact surfaces, but also in processing areas of poultry farms such as walls, floors, pipes, drains and on contact surfaces, such as stainless steel, aluminum, nylon, rubber, plastic, polystyrene, and glass (Joseph et al., 2001; Schonewille et al., 2012; Wang et al., 2013). The adhesion and biofilm-forming ability of this pathogen depend on several factors including the growth medium, the growth

phase of the cells, the type and properties of the inert material, the contact time, the presence of organic material, as well as environmental parameters such as temperature and pH (Speranza et al., 2011).

It is well known that elimination of *Salmonella* biofilm from poultry environment is challenging (Davies & Breslin, 2003; Gradel et al., 2005). In contrast to planktonic microorganisms, biofilm cells have distinctive characteristics such as antibiotic resistance, preservative tolerance and enhanced virulence, leading to chronic infections (Anderson & O'Toole, 2008; Brady et al., 2008; Martinez-Medina et al. 2009; Hancock et al., 2010). Several chemical agents are commercially available for the elimination of *Salmonella*. However, different studies showed high prevalence of *Salmonella* in poultry environment samples after cleaning and disinfecting in broilers and laying hen houses, proving that disinfection was ineffective against the bacteria in a field situation (Davies & Breslin, 2003; Møretrø et al., 2012).

The use of several alternatives to control *Salmonella* biofilm formation, such as enzymes, phagetherapy, extracts from aromatic plants, nanomaterials, quorum sensing inhibitors or bacteriocins have been successfully used (Coughlan et al., 2016; Merino et al., 2019). Lactic acid bacteria (LAB) strains, owing to the production of several antimicrobial components, offer a natural alternative for prevention and control of foodborne pathogens (Marianelli et al., 2010; Sharma et al., 2017; Singh et al., 2018). Recent work has shown that certain LAB strains are able to reduce the formation of biofilms by *Salmonella* spp. (Das et al., 2013; Woo & Ahn, 2013; Chapman et al., 2014; Gómez et al., 2016). This effect could be explained by its ability to coaggregate with potential pathogens and/or produce antimicrobial substances (such as hydrogen peroxide) and bio-surfactants that inhibit bacterial adhesion (Cadieux et al., 2009). Cell surface hydrophobicity, production of extracellular polymeric substances, presence of some structures like fimbriae and flagella and other proteins or polysaccharides, influence attachment to surfaces (Donlan, 2002).

Lactobacillus strains may be used as an alternative to reduce the formation of biofilms by pathogens in the food industries and few studies have investigated the role of lactobacilli on the inhibition of *Salmonella* biofilm formation. The objectives of this work were: 1) to investigate

the ability of different *Salmonella* strains isolated from poultry environment to form biofilms on different surfaces, 2) to determine the relationship between *Salmonella* cell surface properties and biofilm formation, 3) to investigate the effect of three *Lactobacillus* strains and their surface proteins on *Salmonella* biofilm formation.

2. Materials and methods

2.1 Bacterial strains and growth conditions

Fifteen *Salmonella* strains representing three serotypes commonly found in poultry and/or implicated in foodborne disease were selected for testing. Strains were isolated from chickens, poultry compos and eggs (Table 1) and were received from Faculty of Veterinary Sciences (Institute of Veterinary Genetics) and Experimental Agricultural Station (National Institute of Agricultural Technology). Detailed information about recovery method of *Salmonella* was described by others authors (Rodríguez et al., 2018) and the identification was performed by conventional biochemical test and serotyping employing somatic and flagellar antibodies.

Bacteria were stored in stocks cultures at −80 ºC in 20% v/v glycerol. Working cultures were grown for 18 hours at 37°C in aerobic conditions in liquid Luria Bertani (LB; Biokar Diagnostic, Beauvais, France). The *Lactobacillus* strains used for aggregation and inhibition assays were grown in Man, Rogose and Sharpe Broth (MRS; Difco Laboratories, Detroit, MI, USA) at 30°C for 24 or 48 hours in aerobic conditions.

2.2. Hydrophobicity assays for microbial adhesion to hydrocarbons

The protocol was followed according to Golowczyc et al. (2007) with some modifications. Briefly, *Salmonella* cells were harvested in stationary phase by centrifugation for 4 min at 10000 ×g and resuspended in phosphate buffered saline (PBS) (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2). Optical density at 600 nm (OD_{600}) of bacterial suspension was adjusted at 0.3 (OD_{600} = 0.3, equivalent to 8 log CFU/ml) using a spectrophotometer (Metrolab 330, Argentina). Two ml of bacterial suspension were mixed with 0.4 ml of xylene (apolar solvent) by vortexing for 120 s. Since this property is likely affected by the temperature, the measurements were carried out at a controlled temperature (20-22ºC). The phases were allowed

to separate by decantation (5-10 min), and the OD_{600} of aqueous phase was measured. The relative decrease in OD of the aqueous phase was taken as a measure of the cell surface hydrophobicity (%H), as calculated with the formula:

$$\%H = (OD_{600o} - OD_{600})/ OD_{600o} \times 100 \quad (1)$$

where OD_{600o} and OD_{600} are the absorbance before and after extraction with xylene, respectively.

2.3 Autoaggregation assays

Salmonella strains were harvested at stationary phase, collected by centrifugation (10,000 × g for 10 min), washed twice, and resuspended in PBS buffer to OD_{600}=0.3 (8 log CFU/ml). Optical density was measured in a spectrophotometer (Metrolab 330, Argentina) to determine the kinetics of sedimentation. The autoaggregation coefficient (AC) was calculated at 20 h according to Golowczyc et al. (2007) as:

$$AC = [(OD_i - OD_t)/OD_i] \times 100 \quad (2)$$

where OD_i is the initial optical density at 600 nm at time 0 of the microbial suspension and OD_t is the optical density at time *t*.

2.4 Coaggregation assays

Salmonella cells were harvested in stationary phase by centrifugation for 4 min at 5,000 × g and resuspended in PBS buffer. Lactobacilli suspensions cells were obtained similarly from stationary phase culture in MRS. One millilitre of *Lactobacillus* suspension (2×10^8 CFU/ml) and 1 ml of *Salmonella* suspension (2×10^8 CFU/ml) were mixed in glass test tubes and incubated at 37 °C. Optical density was measured at zero time (OD_i^{S-L}) and after 20 hours without shaking the glass tube (OD_t^{S-L}). Coaggregation coefficient (CC) was calculated at time (*t*) according to Golowczyc, Mobili, Garrote, Abraham, & De Antoni (2007) as:

$$CC = [(OD_i^{S-L} - OD_t)^{S-L}/OD_i^{S-L}] \times 100 \quad (3)$$

where OD_i^{S-L} is the initial optical density at 600 nm of the microbial suspension and OD_t^{S-L} is the optical density at time *t* = 20 hours. Coaggregation coefficient (CC) was calculated as stated before but subtracting the corresponding aggregation coefficient (AC) of each strain obtained as explained in section 2.3.

2.5 Surface proteins extraction

Lactobacillus surface protein extraction was performed with 5 M-LiCl according to Golowczyc et al. (2007). *Lactobacillus* cultures were centrifuged for 10 minutes at 10000 *g* in Sorvall centrifuge, washed twice with PBS and the pellet resuspended in 5 ml of 5M LiCl (J.T. Baker, Mallinckrodt Baker S.A., Mexico). The suspension was vortexed for 60 seconds at maximum speed and centrifuged at 5000 g for 10 minutes. Supernatant were dialysed (dialysis membrane of 14 KDa molecular weight cut-off, Sigma) exhaustively for 48 hours against PBS (pH 7.2) under gentle agitation. The PBS was renewed approximately every 12 hours. The protein profiles extracts was tested using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% separating and 4% stacking gels using the discontinuous buffer system in a BioRad Mini-Protean II (BioRad Laboratories, Richmond, CA, USA) equipment. Protein concentration was determined by the Bradford method (Golowczyc et al., 2007). Protein suspensions extracted were lyophilized and stored at -20 °C

2.6. Biofilm Assay

Biofilm assays were performed in 24-well plates and biofilm formation was evaluated by colorimetric method using crystal violet staining, as described by Stepanović et al. (2000) and Bhowmick et al. (2011) with some modifications. Briefly, 1 ml of LB broth was placed in 24-well plates and 10 µl (1%) of the working culture (stationary phase) of different *Salmonella* strains were inoculated in each well. After incubation for 48 hours at 28 °C, the medium was discarded and wells were washed twice with PBS to remove non adhered cells and air dried for one hour to allow fixation of the adhered cells. Then 500 µl of 0.2% crystal violet solution (in PBS) were added per well and incubated at room temperature for 30 minutes. Next, the dye was discarded followed by 3 washings with PBS to remove the excess of dye. The crystal violet from biofilm staining was extracted with 300 µl of 96 % ethanol for 30 minutes. Aliquots (150 µl) of the supernatant was transferred to 96-well plate and the absorbance at 595 nm in a microplate reader (Biotek Synergy HT) was measured. When viable cells enumeration was required, supernatants were removed and biofilm were washed twice with PBS. Aliquots of 1

ml of PBS were added per well and cells were detached by scraping. Serial dilution (1/10) and plate count on LB agar of bacterial suspension was made.

Biofilm assays were performed using three types of surfaces widely used in poultry processing environments: 1-Glass (13 mm diameter and 0.1 mm thickness); 2- Stainless steel sheet (type 304, no. 4 finish, 13 mm diameter and 0.3mm thickness); 3- Polystyrene (16 mm diameter). The test was carried out in triplicate and three independent tests were performed. LB media without inoculations were used as a negative control.

To classify the *Salmonella* strains according to their capacity to form biofilm, the criteria described by Stepanović et al. (2004) and Bhowmick et al. (2011) were used. The cut-off OD value (ODc) was defined as 3 standard deviations above the OD average of the negative control. *Salmonella* strains were classified as follows: O.D. ≤ O.D.c corresponded to non-biofilm producer, O.D.c < O.D. ≤ (2 x O.D.c) to weak biofilm producer, (2 x O.D.c) < O.D. ≤ (4 x O.D.c) represents moderate biofilm producer and (4 x O.D.c) < O.D. corresponded to strong biofilm producer.

2.7. Effect of Lactobacillus and surface proteins on Salmonella biofilm

Inhibitory effect of *Lactobacillus* strains and LiCl-extracted proteins on the *Salmonella* serovar Enteritidis 115 biofilm formation was determined *in vitro* using the commonly used micro-titre plate method (MTP) and the viable count of biofilm cells in LB agar as described above. Two different types of experiments were performed using bacteria or their protein extract: 1) 24-well polystyrene plates were first **preincubated** with lactobacilli (2×10^8 CFU/ml) or surface proteins (0.5 µg/ml) suspension for 2 h and washed softly three times with PBS. Then, 1 ml of BHI medium and 10 µl of *Salmonella* suspension (2×10^8 CFU/ml) was added to each well and incubated for 48 h at 28 ºC. 2) One ml of BHI medium was placed in 24-well polystyrene plates. Then, 10 µl of lactobacilli (2×10^8 CFU/ml) or surface proteins (0.5 µg/ml) and 10 µl of *Salmonella* (2×10^8 CFU/ml) suspension was added to each well and **coincubated** for 48 h at 28 °C. As a negative control, PBS was placed in each well instead of lactobacilli or surface proteins. In both cases, *Salmonella* biofilm formation was studied as described above. None of

the three lactobacilli strains were able to grow in LB agar medium. Each treatment was performed in duplicate in two independent experiments.

2.8. Confocal Microscopy

Biofilms were established on Thermanox glass of 1 cm^2 as described above (for 48 hours at 28 °C) and were staining with the Baclight ™ bacterial viability kit from LIVE / DEAD®. After that, the samples were washed three times with 2 ml of distilled water and were stained with propidium iodide (PI) and SYTO 9 for 10 minutes, to differentiate between living and dead cells, according to manufacturer's instructions.

2.7 Statistical Analysis

Results were expressed as the mean and standard error of at least three independent replicates. Significant differences associated to treatment were analyzed using one-way analysis of variance and Tukey test. The statistical difference was indicated at $p < 0.001$. The Pearson correlation test was used to analyze the biofilm correlation level between hydrophobicity and biofilm formation.

3. Results and discussion

3.1 Surface properties

The interaction between surface and bacterial surface is mediated by a complex array of chemical and physical interactions and there are multiple factors involved in cell attachment such as hydrophobicity, surface conditioning, surface charge, growth medium, etc. As a result, it is difficult to find a relationship between bacteria surface properties and the capacity to form biofilm (Palmer et al., 2007). Di Bonaventura et al. (2008) reported a connection between hydrophobicity of cell surface and *Listeria* strains attachment, colonization, and biofilm formation. However, other authors reported that there is no relationship between aggregation or hydrophobicity and inhibition of pathogen biofilm formation (Gómez et al., 2016; García-Cayuela et al., 2014). Bacterial adhesion to hydrocarbons has been widely used for measuring cell surface hydrophobicity. In this study, cell surface hydrophobicity was measured by xylene extraction, an apolar solvent (Table 2). Most strains were hydrophilic, showing a percentage of

adhesion to xylene between 0 and 7 % indicating low hydrophobicity. Besides, SE 112, 113, 114, 115 and 116 were moderately hydrophobic showing the highest affinities for this solvent (%H =22-38%).

Aggregation abilities of *Salmonella* strains were studied by spectrophotometric assays after 20 hours (Table 2). In general, *Salmonella* strains showed high aggregation abilities (27–54 %), except SG 110 that showed very low values (16 %). Given that most bacteria live in environments with fluctuating conditions (e.g. shear forces, physiological conditions, nutrient availability), the microorganisms within aggregated communities will survive and proliferate under conditions that reduce the prevalence of single non-aggregated cells (Rickard et al., 2003). In addition, the aggregation abilities could be important factors that interfere with the capability of the pathogens to adhere to different surface through competitive exclusion (Collado et al., 2008). The results obtained indicated that strains SE 106 and ST 102 and 108 could have an advantage to biofilm formation.

The coaggregation abilities between *Salmonella* strains and *Lactobacillus plantarum* 83114 and *L. kefiri* 8321 and 83113 strains isolated from kefir grains were very variable (Table 2). Hereof, the highest co-aggregation values were exhibited by ST 108 and SG 110 (more than 72 %). All *Salmonella* strains tested showed coaggregation abilities with the lactobacilli strains tested, but the percentage of co-aggregation was demonstrated to be strain-specific.

Bacterial co-aggregation has a considerable significance in several ecological niches. It has been suggested that co-aggregation abilities of *Lactobacillus* strains might interfere with the ability of the pathogenic bacteria to infect the host and can prevent the colonization of foodborne pathogens (García-Cayuela et al., 2014). In addition, during co-aggregation, *Lactobacillus* strains could control a microenvironment around the pathogens (increasing the concentration of inhibitory substances) or could be involved in a competitive exclusion mechanism which contributes to the reduction of the pathogenic load during infections (Kaewnopparat et al., 2013). Beganović et al. (2011) have reported that surface proteins of *L. helveticus* M92 are involved in the autoaggregation and coaggregation with *S.* Typhimurium.

3.2 Biofilm formation

The results of biofilm formation by *Salmonella enterica* serotypes on different surfaces are shown in the Figure 1. Glass, steel and polystyrene were selected for the study of *Salmonella* biofilm formation because of their extensive use in the food processing industry.

The results of the biofilm formation on glass surface indicated that 47 % of the strains (7 out of 15) were biofilm formers. SG 110 and 109, ST 108, and SE 114, 116 and 105 were weak biofilm formers and only strain SE 112 exhibited a moderate biofilm formation. The rest of the strains were categorized as non-biofilm producers on glass surface.

The results of the biofilm formation on stainless steel surface indicated that 27 % of the strains (4 out of 15) were biofilm formers. SG 109, ST 108, 114 and 105 were weak biofilm formers while the other strains were non-biofilm producers in stainless steel.

More specifically, in the case of glass surface the attached and biofilm cell population of different enteropathogenic strains was found to be lower than on stainless steel surface (Gkana et al., 2017). Stepanović et al. (2004) investigated the capacity of *Salmonella* to produce biofilm on polystyrene, using 122 strains of *Salmonella* isolated from different sources and cultured in different media and reported that only 1.6 % of the strains produced a strong biofilm at 28 ºC in BHI broth. De Oliveira et al. (2014), under the same conditions, observed that 1.7 % of strains were able to form biofilms on stainless steel and glass, but were non-biofilm producers on polystyrene plates. In our study we found that on polystyrene surface, ST 103 and 102 and SE 112 and 107 are weak biofilm formers (27 %). SG 109, SE 113 and 105 exhibited a moderate biofilm formation ability (20 %). Nevertheless, strain SE 115 showed a marked biofilm formation on this surface. Among all the strains studied, strain SE 115 was moderately hydrophobic and presented intermediate autoaggregation values (Table 2). It has often been challenging to demonstrate a clear relationship between the surface properties and biofilm formation in pathogenic bacterial strains because modification in incubation conditions influences the ability of microorganisms to form biofilms (Giovannacci et al., 2000). The relationship between surface hydrophobicity and the amount of biofilm of bacteria has been studied, and the correlations between them are sometimes, but not always evident. Di Bonaventura et al. (2008) observed a positive correlation between the hydrophobicity of *Listeria*

monocytogenes and biofilm formation on glass. Similarly, Wang et al. (2013) observed a positive correlation between cell surface hydrophobicity and the capacity of individual *Salmonella* strains to form biofilms. There was no correlation between bacterial hydrophobicity and biofilm formation in this study (Pearson's correlation coefficient R-square was 0.088, $p > 0.1$). The polystyrene is a hydrophobic surface that favors bacterial adhesion, whereas microorganisms are less likely to adhere to hydrophilic surfaces, such as stainless steel and glass (Simões et al., 2008). Joseph et al. (2001) also observed high *Salmonella* biofilm production on plastic than on stainless steel. In this study, *S.* Enteritidis 115 showed higher biofilm formation on polystyrene ($p < 0.001$) than on glass or stainless steel surfaces.

3.3 Effect of *Lactobacillus* strains and surface proteins on *Salmonella* biofilm

The results presented in Figure 1 indicate that among the *Salmonella* strains, SE 115 exhibited the greatest capability to form biofilm on polystyrene surface. We therefore decided to use this strain to study the inhibition of biofilm formation by *Lactobacillus* strains. Figure 2 shows the inhibition of SE 115 biofilm formation by *Lactobacillus kefiri* strain 8321 and 83113 and *L. plantarum* 83114 isolated from kefir grain. When the three lactobacilli strains were preincubated in the plate (24 h at 37 °C) and after that *Salmonella* strain was added, the results showed that SE 115 biofilm formation was significantly decreased compared to with the control ($p < 0.001$) (Fig. 2A). Similarly, when the three lactobacilli strains were coincubated in the plate (48 h at 28 °C) with SE 115, biofilm formation of this strain was significantly decreased ($p < 0.001$) compared to with the control (Fig. 2B). Inhibition of SE 115 biofilm formation by the *L. kefiri* strains studied was greater (approx. 1 logarithm of reduction) when strains were preincubated in the plate. However, only *L. kefiri* 83113 strain showed significantly greater inhibition of *Salmonella* biofilm formation after preincubation assay ($p < 0.001$). This result could indicate that the lactic bacteria adhere (or even form biofilm) on the plate and this could be the mechanism to inhibit the development of the biofilm by *Salmonella*.

According to several authors, some lactic acid bacteria are able to reduce biofilms formation by *Salmonella* spp. (Chapman et al., 2014; Das et al., 2013; Gómez et al., 2016; Woo & Ahn, 2013). Das et al. (2013) reported that *Lactobacillus plantarum* strain KSBT 56, isolated from a

traditional food product of India, effectively inhibited the growth, invasion and biofilm forming ability of *Salmonella* ser. Enteritidis. Gómez et al. (2016) demonstrated the use of potential probiotic LAB biofilms for the control of *Listeria monocytogenes, S.* Typhimurium and *E. coli* O157:H7 biofilm formation through exclusion mechanisms.

Lactic acid bacteria are recognized to produce a wide range of antibacterial compounds including organic acids, ethanol, diacetyl, hydrogen peroxide (Arena et al., 2017; Camargo et al., 2018). These compounds drastically decrease the pH and are able to inhibit the growth (and even cause death of the cells) of *Salmonella* and affect the formation of biofilm by this pathogen. In addition, the coaggregation capacity with potential pathogens and/or production of antimicrobial substances (such as hydrogen peroxide) and bio-surfactants could inhibit bacterial adhesion (Cadieux et al., 2009). *S.* Enteritidis 115 showed a high percentage of coaggregation with *L. kefiri* 83113 (57 %) and a lower percentage with *L. kefiri* 8321 and *L. plantarum* 83114 (Table 2). However, the three *Lactobacillus* strains were able to produce a significant reduction in *Salmonella* biofilm formation in both pre-incubation and co-incubation assays. These results indicate that there is no relationship between coaggregation and biofilm formation of *Salmonella*. It is important to highlight that the lactobacilli strains interfered with *Salmonella* biofilm formation in *in vitro* conditions (under optimal and static growth conditions for lactobacilli strains). In the food environment the low nutrient content and adverse temperature conditions could be decreased lactobacilli growth and this would be a limitation for the use of this strategy.

Figure 3 illustrates the inhibition of SE 115 biofilm formation by surface proteins extracted from *Lactobacillus kefiri* strain 8321 and 83113 and *L. plantarum* 83114. When the surface proteins extracted from lactobacilli strains were preincubated in the plate (2 h at 37 °C) and after that *Salmonella* strain was added, the results showed that SE 115 biofilm formation was significantly decreased compared to with the control ($p < 0.001$) (Fig. 3A). Similarly, when surface proteins extracted from lactobacilli strains were coincubated in the plate (48 h at 28 °C) with SE 115, biofilm formation of this strain was significantly decreased ($p < 0.001$) compared with the control (Fig. 3B). The S-layer is a proteinaceous envelope constituted by subunits that

self-assemble to form a two-dimensional lattice that covers the surface of different species of Bacteria and Archaea, and it could be involved in cell recognition of microbes among other various functions (Sára & Sleytr, 2000). The presence of S-layers has been described in many bacterial species, including some of the genus *Lactobacillus* (Hynönen & Palva, 2013). Previously, it has been reported that *L. kefiri* isolates from kefir grains have an S-layer and the preincubation of *Salmonella* with this proteins leads to changes in the surface of *Salmonella* thus antagonizing invasion of cultured human enterocytes (Golowczyc et al., 2007). Similarly, these changes in the SE 115 surface in contact with S-layer proteins could cause inhibition of biofilm formation by *L. kefiri* strains. On the other hand, *L. plantarum* 83114 does not have S-layers proteins and surface proteins extracted from this strain produced a significant inhibition, similar to that observed with the S-layer of *L. kefiri*. Other authors have published similar results with surface proteins of other lactobacilli. Petrova et al. (2016) reported that isolated lectin-like molecules from probiotic strain *Lactobacillus rhamnosus* GG possess a pronounced inhibitory activity against biofilm formation by various pathogens, including clinical *Salmonella* species.

The use of confocal laser scanning microscopy may contribute to a complete understanding of the mechanism of inhibition of biofilm formation. Figure 4 shows the biofilm formed by SE 115 on polystyrene and in coincubation experiments with *L. kefiri* strain 8321 and 83113 and *L. plantarum* 83114. The biofilm formed by SE 115 was homogenous with most of the living cells (green). However, when SE 115 was coincubated with the three lactobacilli strains, the biofilm was fully disrupted. These images confirmed the results found by other methodologies and it was observed that SE 115 in the presence of lactobacilli strains decreases its ability to form biofilm. These results indicate that LAB prevents the development of biofilm either through exclusion mechanisms or production of antibacterial compounds that interact with the pathogen or components of the biofilm matrix.

4. Conclusions

There are few studies that have extensively investigated *Lactobacillus* interferences associated with *Salmonella* biofilms and even less their surface proteins. *Lactobacillus* strains isolated from kefir grains could be applied as protective bacteria able to shield different surfaces from the occurrence of *Salmonella* biofilms. Our results show that three *Lactobacillus* strains isolated from kefir grain can be excellent candidates to prevent *Salmonella* biofilm formation. Our results also showed that surface proteins contribute to the *Salmonella* biofilm inhibition. These strains and their surface proteins can be excellent candidates to use as a potential alternative to the application of disinfectants for surfaces and antibiotics in human and animal health as well as in industry worldwide.

Acknowledgments

The authors gratefully acknowledge the financial support provided by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Argentina (PIP 0636), Universidad de La Plata (UNLP), Agencia Nacional de Promoción Científica y Tecnológica (PID 0049) and CYTED Program (Network 115 RT0488). L.M., F.T and M.G. are members of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and GDA is member of the Commission for Scientific Research (CICPBA, Argentina) and profesor at the Facultad de Ciencias Exactas, Universidad Nacional de La Plata.

Legend of the figures

Figure 1. Biofilm formation by *Salmonella enterica* serotypes on different surfaces A. Glass B. Stainless steel C. Polystyrene. The strains were classified as strong, moderate, weak or no biofilm formers.

Figure 2. Biofilm formation by *Salmonella* Enteritidis 115 on polystyrene plates in presence of *Lactobacillus kefiri* strain 8321 or strain 83113 or *Lactobacillus plantarum* strain 83114 in (A) preincubations assays and (B) coincubation assays. Different letter implied significant difference ($p < 0.001$).

Figure 3. Biofilm formation by *Salmonella* Enteritidis 115 on polystyrene plates in presence of surface proteins extracted from *Lactobacillus kefiri* strain 8321 or 83113 or *L. plantarum* 83114 in (A) preincubations assays and (B) coincubation assays. Different letter implied significant difference ($p < 0.001$).

Figure 4. Confocal Laser Scanning Microscopy images of biofilm formation by *Salmonella* Enteritidis 115 on polystyrene plates. (A) *S.* Enteritidis strain 115 (control), (B) coincubated with *L. kefiri* 8321, (C) coincubated with *L. kefiri* 83113, (D) coincubated with *L plantarum* 83114. Magnifications 63X.

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Table 1. *Salmonella* strains used in this study and their source

***Salmonella* serotype**	**Strains**	**Origin**
Enteritidis [a]	SE 105	Chicken
Enteritidis [a]	SE 106	Chicken
Enteritidis [a]	SE 107	Chicken
Enteritidis [b]	SE 112	Chicken
Enteritidis [b]	SE 113	Chicken

Enteritidis [b]	SE 114	Poultry compos
Enteritidis [b]	SE 115	Poultry compos
Enteritidis [b]	SE 116	Poultry compos
Typhimurium [a]	ST 102	Chicken
Typhimurium [a]	ST 103	Chicken
Typhimurium [b]	ST 108	egg
Gallinarum [a]	SG 104	Chicken
Gallinarum [a]	SG 109	Chicken
Gallinarum [a]	SG 110	Chicken
Gallinarum [a]	SG 111	Chicken

[a] Strains received from Gerardo A. Leotta, Faculty of Veterinary Sciences -UNLP, IGEVET-Institute of Veterinary Genetics (UNLP-CONICET LA PLATA).

[b] Strains received from EEA INTA: Experimental Agricultural Station, National Institute of Agricultural Technology.

Table 2. Hydrophobicity (%) or H (%), Autoagreggation coefficient (%) or AC (%), Coagreggation coefficient (%) or CC (%) of *Salmonella* strains.

***Salmonella* strains**	**H (%)**	**AC (%)**	**CC (%) with *Lactobacillus* strains**		
			8321	**83113**	**83114**
SE 105	0.3 ± 1.3	30.5 ± 0.7	15.2 ± 0.4[a]	46 ± 18[b]	28.5 ± 0.5[a]
SE 106	1.9 ± 2.8	54.1 ± 0.7	24 ± 12[a]	69 ± 11[b]	43 ± 3[c]
SE 107	0.2 ± 3.1	29.3 ± 2.0	15 ± 2[a]	59 ± 13[b]	32 ± 5[c]
SE 112	27.9 ± 3.31	27.9 ± 3.3	11.9 ± 0.2[a]	71 ± 3[b]	34 ± 1[c]
SE 113	29.4 ± 3.5	27.7 ± 2.2	8 ± 6[a]	57 ± 2[b]	22 ± 1[c]
SE 114	26.9 ± 9.4	28.8 ± 0.3	8 ± 3[a]	63 ± 1[b]	28.1 ± 0.2[c]
SE 115	22.5 ± 4.1	34.3 ± 3.1	21 ± 5[a]	57 ± 3[b]	34 ± 0.5[a]

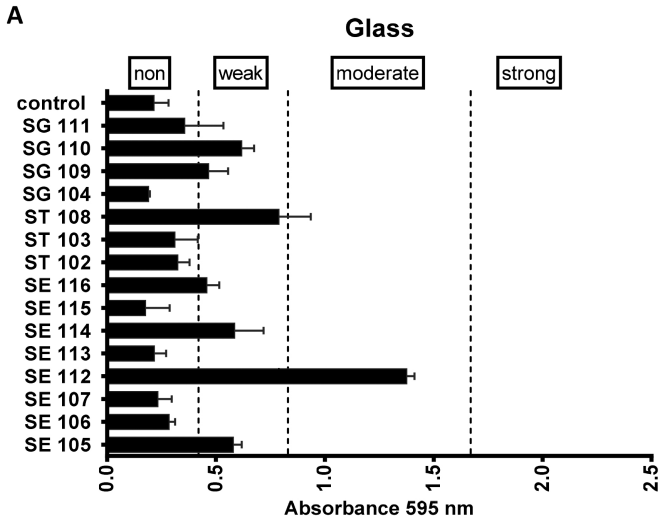
SE 116	38.2 ± 7.1	32.5 ± 0.5	11 ± 3[a]	42 ± 12[b]	35.1 ± 0.8[b]
ST 102	0.6 ± 1.5	45.2 ± 4.3	25 ± 2[a]	66 ± 5[b]	37 ± 4[a]
ST 103	3.1 ± 4.6	33.9 ± 7.4	25 ± 4[a]	72 ± 6[b]	37 ± 1[a]
ST 108	0.3 ± 2.9	42.4 ± 3.6	42 ± 3[a]	90.4 ± 0.6[b]	51 ± 1[a]
SG 104	2.3 ± 2.0	28.1 ± 6.7	5.8 ± 0.4[a]	68 ± 15[b]	30.5 ± 0.4[c]
SG 109	7.2 ± 7.9	26.0 ± 1.3	10 ± 1[a]	55.5 ± 0.6[b]	27 ± 3[c]
SG 110	0.9 ± 6.1	16.2 ± 0.8	50 ± 3[a]	71.7 ± 0.8[b]	27.1 ± 0.9[c]
SG 111	3.2 ± 3.3	28.4 ± 6.3	38 ± 2[a]	73 ± 1[b]	28 ± 1[a]

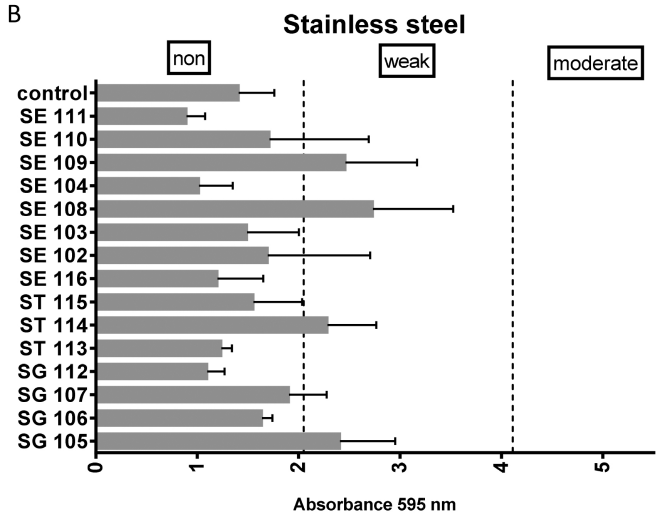
[a,b,c]: Different letters indicate significant differences between the CC (%) results with three *Lactobacillus* strains at alpha 0.05 by Tukey's multiple comparisons

Graphical abstract

Highlights

- Biofilm formation of *Salmonella* isolated from poultry was investigated under different conditions.
- Three *Lactobacillus* strains can be excellent candidates to prevent *Salmonella* biofilm formation.
- Surface proteins extracted from lactobacilli strains contribute to the *Salmonella* biofilm inhibition.


A
Glass
non
weak
moderate
strong
control
SG 111
SG 110
SG 109
SG 104
ST 108
ST 103
ST 102
SE 116
SE 115
SE 114
SE 113
SE 112
SE 107
SE 106
SE 105
0.0
0.5
1.0
1.5
2.0
2.5
Absorbance 595 nm


B
Stainless steel
non
weak
moderate
control
SE 111
SE 110
SE 109
SE 104
SE 108
SE 103
SE 102
SE 116
ST 115
ST 114
ST 113
SG 112
SG 107
SG 106
SG 105
0
1
2
3
4
5
Absorbance 595 nm

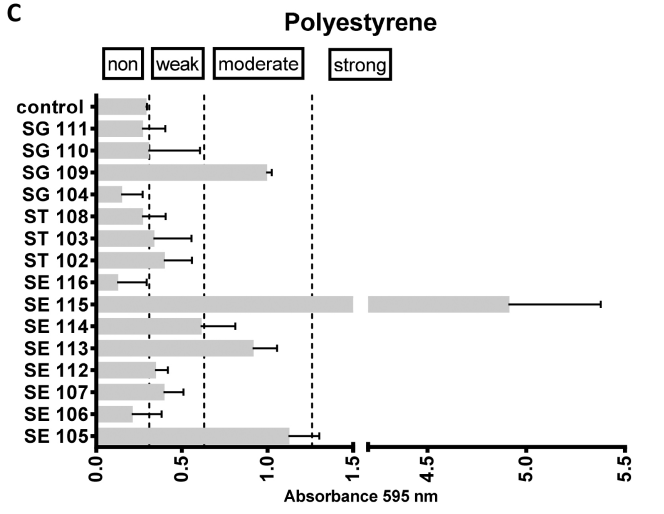
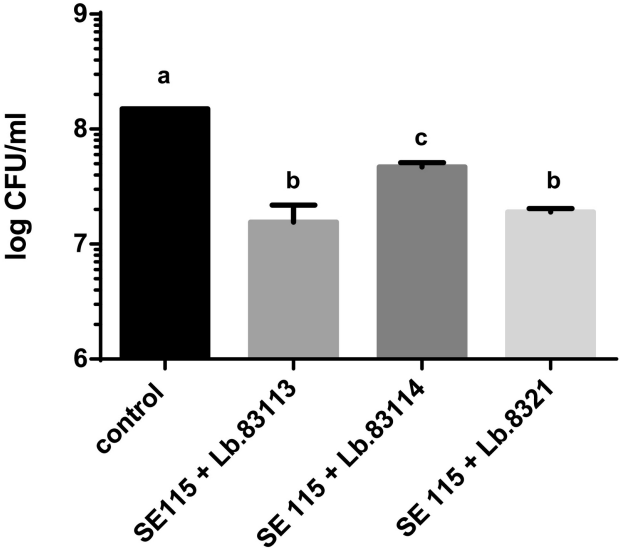

C
Polyestyrene
non
weak
moderate
strong
control
SG 111
SG 110
SG 109
SG 104
ST 108
ST 103
ST 102
SE 116
SE 115
SE 114
SE 113
SE 112
SE 107
SE 106
SE 105
0.0
0.5
1.0
1.5
4.5
5.0
5.5
Absorbance 595 nm

Figure 1

A.


log CFU/ml
9
8
7
6
a
b
c
b
control
SE115 + Lb.83113
SE 115 + Lb.83114
SE 115 + Lb.8321

B.

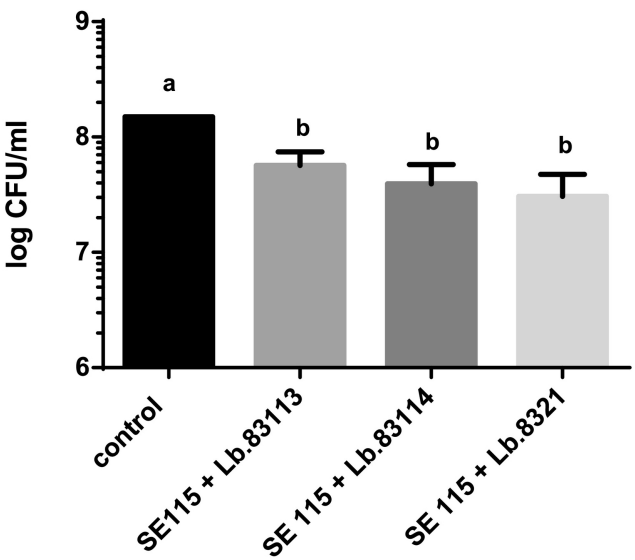

log CFU/ml
9
8
7
6
a
b
b
b
control
SE115 + Lb.83113
SE 115 + Lb.83114
SE 115 + Lb.8321

Figure 2

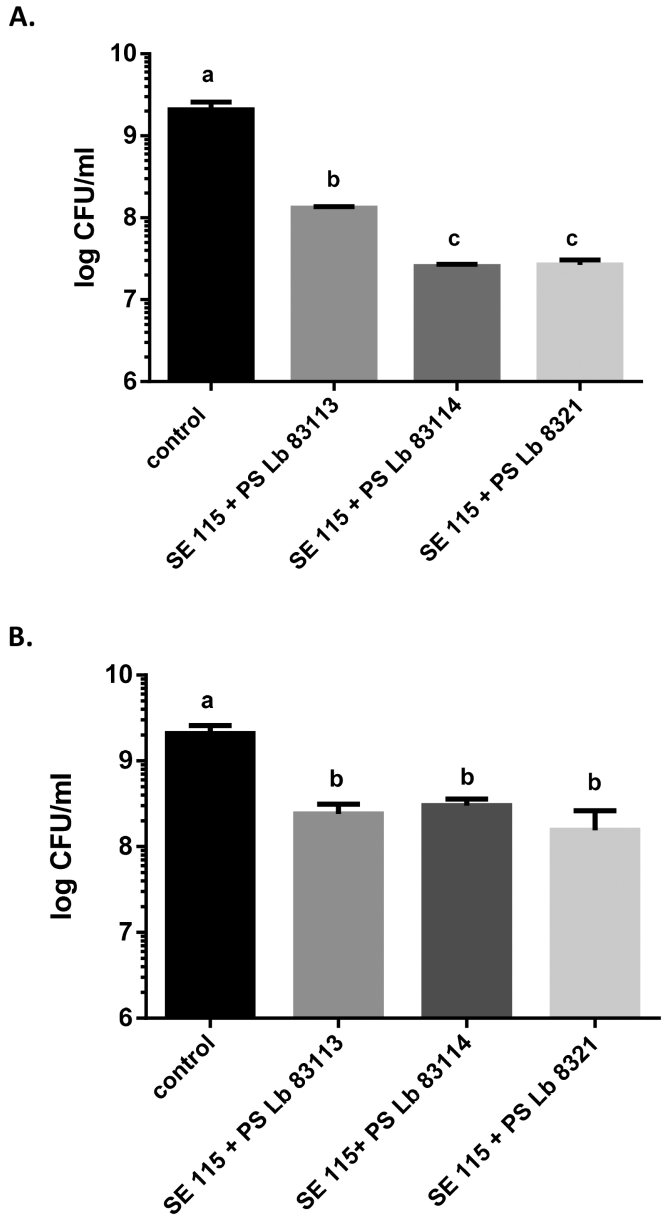

A.
log CFU/ml
10
9
8
7
6
a
b
c
c
control
SE 115 + PS Lb 83113
SE 115 + PS Lb 83114
SE 115 + PS Lb 8321
B.
log CFU/ml
10
9
8
7
6
a
b
b
b
control
SE 115 + PS Lb 83113
SE 115+ PS Lb 83114
SE 115 + PS Lb 8321

Figure 3

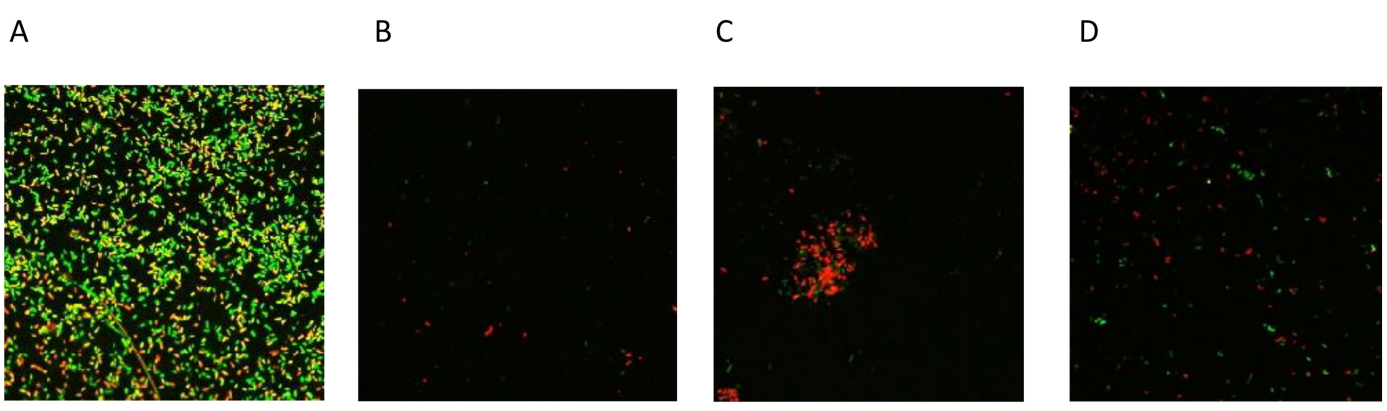


Figure 4